Imprinting of \textit{RB1}
(the new kid on the block)

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Abstract
Recent data have revealed that the paradigmatic tumour suppressor gene \textit{RB1} on chromosome 13 is preferentially expressed from the maternal allele. Imprinted expression of \textit{RB1} is linked to a differentially methylated CpG island in intron 2 of this gene (CpG 85). On the paternal chromosome, CpG 85 is unmethylated and acts as a weak promoter of an alternative \textit{RB1} transcript. Paternal mRNA levels are probably reduced as the result of transcriptional interference of the regular promoter and the alternative promoter on this chromosome. CpG 85 is part of a truncated processed pseudogene (\textit{KIAA0649P}) that integrated into the \textit{RB1} gene prior to the speciation of extant primates. It is plausible that differential penetrance and variation of age at diagnosis, which have been observed in patients with hereditary and non-hereditary retinoblastoma, respectively, are a consequence of imprinted expression of the \textit{RB1} gene. Interestingly, \textit{RB1} is imprinted in the same direction as \textit{CDKN1C}, which operates upstream of \textit{RB1}. The imprinting of two components of the same pathway indicates that there has been strong evolutionary selection for maternal inhibition of cell proliferation.

Keywords: \textit{RB1}; imprinting; DNA methylation

DISCOVERY OF AN IMPRINTED CpG ISLAND IN THE RB1 GENE
In diploid organisms, usually both alleles of a gene are active or inactive. In placental mammals, however, a small subset of genes is imprinted and expressed in a parent-of-origin dependent manner. Imprinting is an epigenetic process leading to parent-of-origin specific DNA methylation and gene expression. Because of experimental limitations, the identification of imprinted genes in humans is challenging. Kanber \textit{et al.} [1] have identified a novel imprinted locus by genome wide CpG methylation analysis of DNA from blood in a patient who was hypomethylated at all known imprinted loci. By this, a 1.2 kb CpG island inside intron 2 of the human retinoblastoma gene (\textit{RB1}) was found to show parent-of-origin specific methylation—it is methylated on the maternal chromosome 13 and unmethylated on the paternal chromosome 13. This CpG island (CpG 85) serves as a promoter for an alternative transcript of the human retinoblastoma gene (\textit{RB1}), which is expressed from the unmethylated paternal chromosome only (Figure 1). The first exon of this alternative transcript is E2B, which is spliced onto exon E3. This feature distinguishes CpG 85 from two other CpG islands associated with the \textit{RB1} gene: CpG 42, which is located a few kilobasepairs upstream of CpG 85, is biallelically methylated, whereas CpG 106, which overlaps the \textit{RB1} promoter and exon E1, is biallelically unmethylated. Greger \textit{et al.} [2] were the first to show that CpG 106 is methylated in some retinoblastomas. This was the first hint that promoter methylation of a tumour suppressor gene plays a role in tumorigenesis.
EVOLUTION OF CpG 85
CpG 85 is part of a 4.5 kb 5’ truncated processed pseudogene derived from the protein coding gene KIAA0649 on chromosome 9 that was integrated in the RB1 locus in reverse orientation (Figure 2). An independent retrotransposition event has lead to the existence of four additional truncated processed pseudogenes of KIAA0649 located in close proximity on chromosome 22q11.21. The open reading frame, which is located in exon 4 of the ancestral gene, is lost in all five processed copies. In contrast to the chromosome 13 copy, no evidence for genomic imprinting was found for the chromosome 22 integrated sequences.

The four small (<300 bp) CpG islands (CpG 19, CpG 17, CpG 26 and CpG 19) present in exon 4 of KIAA0649 are not present in the pseudogene copies on chromosome 22. In the chromosome 13 pseudogene copy (KIAA0649P), however, these CpG islands correspond to two big CpG islands CpG 85 and CpG 42 (Figure 2). Thus, it appears that the human CpG 85 has evolved from two small CpG islands in the ORF of KIAA0649. However, it is also possible that an originally big CpG island in the ancestral locus was maintained in KIAA0649P, but deteriorated in the ancestral gene as well as in the chromosome 22 pseudogene copies after the retrotransposition events.

In contrast to CpG 42, which is completely methylated, CpG 85 has acquired differential methylation after retrotransposition leading to allele specific methylation and monoallelic expression of the alternative RB1 transcript. KIAA0649P and the CpG island are also present in the RB1 gene of other primates (chimp, orangutan, macaque) and New World monkeys (marmoset), but not in the RB1 gene of mice and rat (for details see [1]).

ALLELIC EXPRESSION IMBALANCE OF RB1 TRANSCRIPTS
If the alternative transcript (RB1) were expressed independently of and in addition to the regular paternal RB1 transcript, then the total level of paternal transcripts should be higher than that of the maternal transcripts. However, analysis of parent-of-origin dependent expression of RB1 transcripts revealed an ~3-fold excess of maternal RB1 mRNA [1]. In mice, which do not have the intronic CpG island, no parent-of-origin specific expression imbalance was found, indicating that skewed allelic expression of the human RB1 is linked to the differentially methylated CpG 85. This notion was further substantiated by the finding that demethylation of CpG 85 in lymphoblastoid cell lines by 5-aza-2’-deoxycytidine treatment resulted in reduced skewing of the allelic RB1 transcripts, which is to be expected because after loss of CpG 85 methylation the maternal allele resembles the paternal allele [1]. These results show that allele-specific methylation of CpG 85 affects expression of RB1, probably by transcriptional interference.

TRANSCRIPTIONAL INTERFERENCE
Transcriptional interference refers to a mechanism in which the transcription of one gene has a suppressive influence on the transcription of another gene [3]. This suppressive influence is due to the interference of the RNA polymerase II machinery transcribing one gene with transcriptional initiation, elongation or termination at a neighbouring gene. Thus the act of transcription itself rather than the sequence of the transcribed RNA is important.
Transcriptional interference is the most likely mechanism underlying skewed \textit{RB1} expression depending on differential CpG \textit{85} methylation. Possibly, the transcription complex binding to the unmethylated 2B-promoter acts as a roadblock for the regular transcript on the same (paternal) allele resulting in reduced abundance of paternal \textit{RB1} transcripts (Figure 3) \cite{3}.

\textbf{BIOLOGICAL EFFECTS OF \textit{RB1} IMPRINTING}

The findings reported here extend the observations on epigenetically controlled transcriptional interference by retrotransposons \cite{4–6} to include truncated processed pseudogenes and support the notion that genomic imprinting builds on host defence mechanisms \cite{7–10}. Imprinting of \textit{RB1} may explain certain parent-of-origin effects in human phenotypes caused by mutations in the \textit{RB1} gene.

\textbf{Mutations of the \textit{RB1} gene cause predisposition to retinoblastoma and to other tumours}

According to current knowledge, the only phenotypic consequences of \textit{RB1} gene mutations in the human are tumour predisposition and tumour development. Specifically, mutations in this gene are a prerequisite for development of retinoblastoma (Rb).

\textit{Rb} is a malignant tumour of the eye that is almost exclusively diagnosed in young children. In 60\% of patients, the tumour affects one eye only (unilateral \textit{Rb}) while the remaining 40\% of patients have tumours in both eyes (bilateral \textit{Rb}). Most children with bilateral Rb show multiple tumour foci in both eyes. Moreover, patients with bilateral Rb have a high risk to develop other tumours (second cancer) later in life. Almost all patients with sporadic bilateral \textit{Rb} and all patients with familial \textit{Rb} have a hereditary tumour predisposition that is transmitted as an autosomal dominant trait (hereditary \textit{Rb}). Hereditary \textit{Rb} is caused by heterozygous mutations in the \textit{RB1} gene. Tumour development is initiated by a second mutation that inactivates the other allele of the \textit{RB1} gene. Most patients (>85\%) with sporadic unilateral \textit{Rb} have non-hereditary \textit{Rb}. In these patients, the first and second \textit{RB1} gene mutations are detected in the tumour only.

\textbf{Genotype–phenotype correlations}

Hereditary \textit{Rb} shows variable phenotypic expression. The main parameters of variation are (i) the number of eyes affected (no \textit{Rb}, unilateral \textit{Rb} and bilateral \textit{Rb}), (ii) age at diagnosis and (iii) the development of second tumours later in life. Because of the stochastic nature of the mutational events that are required for tumour development (notably second mutations), phenotypic expression in individual patients is influenced by chance. In addition, phenotypic expression in hereditary \textit{Rb} varies depending on the functional type of the first (i.e. predisposing) mutation in the \textit{RB1} gene:

- \textit{loss-of-function}: most germline \textit{RB1} gene mutations are point mutations that result in premature termination codons (nonsense, frameshift, splice

\begin{figure}
\centering
\includegraphics{Figure2.png}
\caption{Structure of \textit{KIAA0649} and the processed pseudogene \textit{KIAA0649P} on human chromosome 13. The two small CpG islands in exon 4 of \textit{KIAA0649} (CpG 19/CpG 17 and CpG 26/CpG 19) correspond to CpG 85 and CpG 42 in the chromosome 13 copy, respectively. The open reading frame of \textit{KIAA0649} in exon 4 is indicated by the start and stop codons (ATG and TAA) and is lost in \textit{KIAA0649P}. CpG 85 serves as a promoter for an alternative \textit{RB1} transcript (transcript \textit{RB1-2B}). Arrow at CpG 138 and CpG 85, orientation of transcription; arrows between \textit{KIAA0649} and \textit{KIAA0649P} indicate integration of the processed \textit{KIAA0649} gene in intron 2 of the \textit{RB1} gene.}
\end{figure}
mutations causing out-of-frame exon skipping) and trigger nonsense mediated decay. Families segregating a loss-of-function mutation almost invariably show complete penetrance and bilateral Rb.

- **Partial loss-of-function**: patients heterozygous for mutations that do not result in premature termination (regulatory, missense, in-frame) develop fewer Rb foci and families segregating partial loss-of-function mutations often show incomplete penetrance (low-penetrance retinoblastoma).

In non-hereditary Rb the spectrum of first somatic RB1 gene mutations is much the same as the spectrum of RB1 germline mutations. The spectrum of second somatic mutations, however, is distinct in two respects. First, in about 70% of Rb tumours chromosomal mechanisms such as mitotic recombination have led to loss of the normal and thus demasking of the mutant allele (loss of heterozygosity). Second, about 10% of Rb tumours show hypermethylation of the CpG island associated with the regular promoter of the RB1 gene.

**Parent-of-origin effects associated with germline mutations in the RB1 gene**

In order to detect parent-of-origin effects one might wish to compare phenotypic expression between patients who have a new germline mutation on the paternal allele to those patients who have the same functional type of new germline mutation on the maternal allele. However, for reasons that are most likely not associated with imprinting (most notably: differential mutation rate), almost all new RB1 gene mutations arise in the paternal germline (the only known exception being gross deletions). Given this situation, the best source for an analysis of parent-of-origin effects are extended families with several transmissions of the same mutant allele via both sexes.

Survival of patients with retinoblastoma improved only a few generations ago. Therefore, reports of families with retinoblastoma reaching back several generations are rare. In 1960, Macklin [11] reported a retinoblastoma family (pedigree 60 and 63) with remarkable variation of phenotypic expression and penetrance. When analysing this family under the aspect of parental origin, which was not done in the paper by Macklin [11], it appears that phenotypic expression in sibships is more severe if the predisposing allele was transmitted via the father (five bilateral and four unilateral patients among 29 children of fathers at risk and no patient in any of 22 offspring of mothers at risk, Fisher’s exact test two-tailed \( P = 0.00007 \)). When analysing large Rb pedigrees published since then it becomes clear that the parent-of-origin effect seen in Macklin’s Rb family is unusual but not without parallel.

A parent-of-origin effect in two families segregating the same splice site mutation in the RB1 gene, IVS6+1G>T, that causes skipping of exon 6, was reported by Klutz et al. [12]. We found that variation of phenotypic expression was associated with the relative abundance of mutant transcript and with the sex of the parent that transmitted the mutant allele. The regular promoter of the RB1 gene showed no abnormal methylation status in mutation carriers of these families. The direction of the parent-of-origin effect is the same as in the family reported by Macklin: paternally transmitted mutant alleles are associated with a more severe phenotype.

**Figure 3:** The road block model of transcriptional interference. The transcription complex binding to the paternally unmethylated 2B-promoter acts as a roadblock for the transcription complex of the regular transcript on the same allele. Open rectangle, unmethylated promoter; filled rectangle, methylated promoter; dark grey triangle, transcription complex of the regular RB1 transcript; light grey triangle, transcription complex of the RB1-2B transcript.
Since then, additional families segregating the IVS6+1G>T mutation have been identified. In all, nine families are known. In these families, a total of 38 individuals have inherited the mutant allele from their fathers and 22 individuals have inherited the mutant allele from their mothers. In summary, of the 38 individuals with mutant alleles of paternal origin 18 (47%) developed bilateral Rb and 9 (24%) showed unilateral Rb. The remaining 11 (29%) stayed free of Rb. By contrast, no one of the 22 individuals with mutant alleles of maternal origin developed bilateral Rb and only two (9%) had unilateral Rb. Most family members heterozygous for a IVS6+1G>T allele of maternal origin did not develop Rb (20/22, 91%). To address the possibility that all these families share a single founder in whom the IVS6+1G>T mutation has occurred on the background of a genetic variation that is required to bring about the parent-of-origin effect we determined the haplotype background in phase with the IVS6+1G>T allele in those families available to us (Lohmann et al., unpublished data). We genotyped a set of sparsely correlated SNPs from the RB1 gene region (rs1981434, rs2227311, rs2854345, rs3092904, rs4151540, rs4151551, rs4151636) [13] in four families. In three families the mutant allele was in phase with the most frequent (66%) European haplotype. However, the haplotype background was distinct in the fourth family (other alleles at rs1981434, rs3092904 and rs4151540). Therefore, common ancestry of the mutant allele is very unlikely and we conclude that the parent-of-origin effect in these families is associated with the IVS6+1G>T base substitution proper. It is to be noted that all known patients with other base substitutions that result in exon 6 skipping (i.e. IVS6+1G>A or >C) have non-familial retinoblastoma and—if parental DNA was available for testing—new germline mutations. Therefore it is unlikely, that the aberrantly spliced RB1 mRNA that lacks exon 6 per se is the cause of the parent-of-origin effect.

Parent-of-origin effects associated with somatic mutations in the RB1 gene

Genetic alterations in sporadic osteosarcoma can involve somatic mutations of the RB1 gene, including chromosomal mechanisms that result in loss of heterozygosity at loci in the region of the RB1 gene. In a series of sporadic osteosarcomas, Toguchida et al. [14] identified 13 tumours with allele loss at the RB1 locus and found that in 12 of them the initial mutation was on the paternal gene ($P=0.03$ Fisher’s exact test). Such a bias suggests that the oncogenic effect of mutations on the paternal RB1 allele is higher compared to that of mutations on the maternal allele. To date, no other reported study has addressed RB1 parent-of-origin effects in osteosarcoma.

In a study designed to identify clinical parameters discriminating patients with hereditary Rb we found that patients with the initial mutation on the paternal RB1 allele had an earlier age at diagnosis than patients with an initial mutation on the maternal allele (median age at diagnosis 482 versus 865 days [15]). When comparing the distribution of age at diagnosis (Figure 2 in [15]) it appears that this difference might be caused by a subgroup of patients with early diagnosis and initial mutation on the paternal allele.

The above-mentioned study [15] included 111 tumours from patients with non-hereditary Rb and known parental origin of the first mutation (excluding whole RB1 gene deletions). In these data a small excess of paternal first mutations is observed (61/111, 55%). We now know the parental origin of allele loss in 171 tumours from patients with sporadic unilateral Rb (Lohmann et al., unpublished data). Of these, 104 (61%) have retained the paternal allele which is a significant departure from an expected 50:50 ratio (likelihood-ratio Chi$^2$ $P<0.0045$) in favour of first somatic mutations on the paternal RB1 allele. This bias suggests that in some Rbs the oncogenic effect of mutations on the paternal RB1 allele is higher compared to that of mutations on the maternal allele.

THE GREATER PICTURE: IMPRINTING AND CELL-CYCLE REGULATION

The RB1 gene is not the only cell-cycle regulatory gene known to be imprinted. Specifically, the CDKNIC gene, which encodes a cyclin-dependent kinase inhibitor operating upstream of the RB1 protein, is maternally expressed [16]. Intriguingly, the imprint imposed on the RB1 gene acts in the same direction. A rough sketch of the pathway is shown in Figure 4. Unphosphorylated RB1 prevents the transcription factor E2F from binding to its target genes, recruits the histone deacetylase HDAC1 to silence them and thus blocks cell-cycle progression. Phosphorylation of RB1 by a cyclin-dependent kinase (CDK) relieves this block by allowing E2F to activate its target genes. CDK is inhibited by
CDKN1C. Thus, both RB1 and CDKN1C block cell-cycle progression. The CDKN1C gene maps to 11p15.5 and is expressed preferentially from the maternal allele. Mutations of the maternal allele account for ~5% of patients with Beckwith–Wiedemann syndrome, which is an overgrowth syndrome. Imprinted expression of CDKN1C is regulated by a differentially methylated region (DMR) within the KCNQ1 gene. The DMR is methylated on the maternal chromosome and unmethylated on the paternal chromosome. It serves as a promoter for a long non-coding RNA (LIT1 or KCNQ1OT1), which is expressed from the paternal allele only. The unmethylated DMR and/or transcription of LIT1 appears to downregulate the paternal allele of CDKN1C by a not yet completely understood mechanism. Similar to RB1, CDKN1C expression is not strictly monoallelic, probably because complete imprinting would make an individual vulnerable to childhood cancer and would thus have been selected against. On the other hand, imprinting of two components of the same pathway (CDKN1C and RB1) indicates that there has been evolutionary selection for maternal inhibition of cell proliferation.

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References

Key Points
- The human retinoblastoma gene (RB1) is imprinted.
- Imprinted expression of RB1 is linked to a differentially methylated CpG island within a truncated processed pseudogene that integrated into the RB1 locus.
- Imprinting of RB1 probably explains parent-of-origin effects in patients with retinoblastoma.
- Imprinting of RB1 indicates that there has been strong evolutionary selection for maternal inhibition of cell proliferation.


